The Effects of Allogeneic cADSCs on an Experimental Ear Auricular Defect to Evaluate Cartilage Regeneration in a Canine Model

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Abstract

Objective: Ear is one of the most important organs of the auditory system and may fail as a result of trauma, tumors, or other diseases. Allograft transplantation of adult stem cells has been used as an excellent procedure for restoring damage from different diseases. The aim of this study is to assess the regenerative ability of adipose-derived stem cells in ear auricle cartilage defects in a dog model injected with different treatments.

Materials and Methods: The adipose-derived stem cells were isolated from canine adipose tissue. After cultured in medium, these cells were obtained after four passages. This study evaluated into three groups: Groups 1 and 2 were determined by mixing of the adipose-derived stem cells with fibrin glue biomaterial and without fibrin glue, respectively. The mixing of the adipose-derived stem cells with fibrin glue biomaterial and without fibrin glue were separately injected into the mid-portion of a surgically provided canine auricular cartilage injury. After two months, the auricular defect evaluated histologically.

Results: Our findings demonstrated that points of new cartilage were formed at the location of the surgically stimulated defect. Four months after the surgical injection, it was shown that the defect was completely ameliorated with mature and native cartilage tissues. Also, there was no observed formation of cartilage tissue in the control group.

Conclusion: Our results clearly indicate that allogeneic adipose-derived stem cells implantation into the experimental ear auricle defect is a safe, effective and relatively simple therapy of ear lesion in dogs, with a significant improvement of ear cartilage regenerative.

Keywords

Allogeneic ADSCs; Fibrin Glue; Ear Auricle Defect; Repair

Abbreviations

ADSC: Adipose-Derived Stem Cells; BMSC: Bone Marrow-Derived Mesenchymal Stem Cells; CD: Clusters of Differentiation; DMEM: Dulbecco's Modified Eagle Medium; FBS: Fetal Bovine Serum; FG: Fibrin Glue; H and E Staining: Haemotoxylin and Eosin Staining; PBS: Phosphate Buffered Saline

Highlights

- Determine the effects of allogeneic cADSCs on an experimental ear auricular defect to evaluate cartilage regeneration
- Engineer an ear cartilage construct that resembles the ear in shape, size and flexibility
- The canine ADSCs containing tissue progenitor cells and can use in cartilage reconstruction

Introduction

Dog is an ideal model for cartilage tissue engineering and has been proposed as an animal model for a broad range of applications in biomedical research, such as for the studies of respiratory diseases, cardiomyopathies, neurological disorders and cartilage lesions [1-9].

Natural reconstruction of cartilage defects has always been challenging because this tissue has low regeneration intrinsic potential [10,11]. Recently, pre-clinical animal experiments showed that adult stem cells are new promising choices that may comfort cartilaginous tissue regeneration in the cartilage injuries and defects [12,13]. These cells have been potential of beneficial cytokines and growth factors secretion for therapeutic purposes. Zuk, et al., in 2011

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demonstrated that Adipose-Derived Stem Cells (ADSCs) are an essential source of adult stem cells [14-16].

Adipose-Derived Stem Cells (ADSCs) are unique mesenchymal cells that are used as tools for therapeutic purposes in regenerative medicine procedures [14-17]. These cells have been suggested as a promising candidate for the regeneration of cartilage defects *in-vivo* [18-20]. Generally, these cells have similar properties as other source of adult stem cells, which are known as Bone Marrow-Derived Mesenchymal Stem Cells (BMSC). Both the cell-types are able to differentiate towards chondrocyte cell and have potential to be used in cartilage defect regeneration [19,21].

Although, isolation methods of ADSCs have little or no donor site morbidity and a shortened operating time as compared with other adult stem cells such as BMSCs [22-24]. Also, ADSCs have several advantages as compared to BMSCs such as easier isolation, low risks during tissue sampling, the higher yield (up to 100 times more stem cells per g of tissue as compared to bone marrow). Recently, the higher capacity of ADSCs in comparison to BMSCs were verified in several clinical studies in animal models [15,23,24].

Tissue engineering is a scientific field which covers cell therapy, biomaterial and cell biology aspects, to improvement the maintenance, repair and replacement of defected and damaged tissues [25]. This is of science has shown tremendous potential to mix stem cells with biocompatible biomaterial in order to facilitate treatment of ear auricle cartilage defects. The utilization of natural biomaterials suggest a number of advantages for cartilage tissue engineering. Natural materials such as fibrin glue has been used for cartilage tissue engineering [26]. At present, fibrin glue is the most popular natural material in cartilage regenerative medicine because of its non-toxicity, non-immunogenic reaction, encapsulation and uniform distribution of cells [27].

The aim of the present study was to examine the effects of allogeneic cADSCs on an experimental ear auricular defect to evaluate cartilage regeneration in canine model.

Methodology

In this study, six healthy adult local dogs were used. All procedures performed in studies involving animal participants were in accordance with the ethical standards of the institutional and/or national research committee. Dogs were separately maintained in several cages in a standard laboratory condition and provided water and food. Studies were done after one month of parasite testing. First, we removed around 10 grams of fat from local dog's body through a small incision in the abdomen. This procedure is performed under general anesthesia and takes around 15 minutes. Also, under general anesthesia and suitable conditions, injection was done and the dissected skin area sutured, according to the principles of veterinary ear surgery. The adipose-derived stem cells were isolated from six-month-old local dogs. Subcutaneous adipose

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tissues in the dog abdomen were removed under sterile conditions. After washing with PBS and removing the adipose tissue membranes and blood vessels, cADSCs were isolated using the collagenase digestion enzyme. The adipose tissues were cut into small segments, placed in falcon tube and digested with 1.5 ml of type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 45-60 min. The reaction was terminated by adding an equal volume of DMEM medium supplemented 10% FBS. Cells were centrifuged at 1800 rpm for 10 min to remove undigested tissues. The lipid layer and supernatant were discarded after centrifugation. Cells were washed with PBS and were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Gr and Isl and, NY, USA) containing 20 % Fetal Bovine Serum and 1% Pen/Strep solution. Cells were then inoculated onto cell culture flasks and incubated in a 37°C, 5 % CO₂ cell culture incubator. Non-attached cells were discarded after one day and medium was replaced after thirty six hours; after that, the culture medium was changed every 3 day. Cells at the third passage were chosen and CD44, CD90, CD45 and CD34 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added separately. After incubation at 37° C for 30 min in the dark, cells were characterized by flow cytometry (BD Biosciences, San Jose, CA, USA).

These cells were cultured *in-vitro* and mixed with Fibrin Glue (FG) biomaterial. Full-thickness defects were produced in the auricle cartilage of both ears in six adult local dogs. Cell/ Fibrin glue composites were injected into the cartilage defects.

Three groups were formed according to injected-cell/biomaterial type: Group A: Canine mesenchymal stem cells/ Fibrin glue; Group B: Canine adipose-derived stem cells alone; Group C: Sterile serum and no cells (as control group). Regenerated tissues were evaluated histologically and molecularly eight weeks after injection.

All the quantitative data in total collagen content were expressed as mean $\pm$ SD (n = 6; two samples were obtained from each canine adipose tissue). One-way analysis of the variance was used to determine the statistical significance between groups and a value of $p < 0.05$ was considered statistically significant.

Results

In this work, stem cells were successfully isolated from the canine subcutaneous fat. After one day in the primary culture, canine adipose-derived stem cells' shapes were changed. This morphology change, from round to spindle shape, was observed using the optical microscope. The passage 3 canine adipose-derived stem cells were cultured in proliferation medium containing DMEM supplemented with 1% Pen/Strep solution and FBS until cells attained a confluence of up to 80%. Flow cytometry demonstrated that canine stem cell markers CD90 and CD44 were present in canine adipose-derived stem cells.

The canine mesenchymal stem cells, isolated from the canine adipose tissue, rapidly spread in the culture medium containing DMEM supplemented with 10% FBS and 1% Pen/Strep antibiotics. After cells arrived at four passage stage, they were mixed with Fibrin glue biomaterial. Also, cells without Fibrin glue were used in the next group in this study.

There was a significant increase in the rate of proliferation on fibrin scaffold in presence of chondrogenic medium. These fibrin hydrogel provide a favorable 3-D environment to accommodate ADSCs at high seeding densities and promote cellular condensation required for neocartilage formation.

The transcript levels of cartilage-related genes including aggrecan, collagen type II and collagen type I were examined by Real-time PCR. Both aggrecan and collagen type II transcript levels were up-regulated, indicating a differentiation trend of chondrocytes. Transplant integration into the defect site was analyzed histologically by H and E staining. Histological findings evaluations demonstrated that a small mass of new formed cartilage tissue was formed at the location of the surgically-created defect (Fig. 1-4). Histological analysis also confirms the presence of chondrogenesis following the 8-week injection period. Areas of intense coloring for collagen type II and aggrecan in the location of the surgically-created defect were evident. The formed cartilage consisted of fresh chondrocytes with bluish lacuna. This new cartilage was collected close together.

Figure 1: The schematic image demonstrates the different stages of the present study.

Figure 2: The. Flow cytometry of cADSCs at passage 3. Note: hADSCs surface markers. By flow cytometry, typical hADSCs surface markers were shown in A) CD44, B) CD90, C) CD34 and D) CD34 and CD45 as negative marker.

Figure 3: The mRNA expression of Collagen Type I, Collagen Type II, Collagen X, Sox9 and Aggrecan in the three group.

Figure 4: Histological evaluation of tissues Regenerated on Three groups (Group A, canine mesenchymal stem cells/ Fibrin glue; Group B, canine adipose-derived stem cells alone; Group C, sterile serum and no cells as control group) at 8th week After injection. Staining of native cartilage used as control with hematoxylin/eosin is demonstrated in (A); Group A (B); Group B and (C) Group C with $\times 10$ magnification (D). ($\times 10$ magnification).

Discussion

Nowadays, the regeneration of cartilage defect is accounted, as an ideal option to treat of cartilage lesions and diseases using tissue engineering and regenerative medicine procedures. Stem cells are non-specialized cells that are found in many adults and embryonic tissues [7,28]. These cells have the highly self-renewal potential and ability to differentiate into many kinds of cell lineages in vitro and *in-vivo* [14,15,21]. Adipose tissue is as one of the significant tissues used to obtain these cells [14]. Adipose-Derived Stem Cells (ADSCs) have the multipotent characteristics, reproducibility and plasticity [14,15]. They also have high repair potential as a promising approach for the treatment of diseases in tissue engineering and regenerative medicine [12,28]. The self-renewal and the multipotency of ADSCs are the importance properties for regenerative medicine purposes. For this reason, the application of adipose-derived stem cell therapy for tissue regeneration and repair has been a rapidly growing area of scientific endeavor in both human and veterinary medicine over the past several years [29-31]. Although, autologous adipose-derived stem cells remains the gold standard for regenerative medicine [31,32], but the proliferation and differentiation of these cells is restricted by age [33]. Due to this limitation, allogeneic adipose-derived stem cells can be used for regenerative medicine [34,35]. Specifically, allogeneic ADSCs isolated from healthy, young donors are the

ideal candidates for cell therapy in animal model research [36,37]. Many studies have reported repair of cartilage using allogeneic ADSCs and various types of scaffolds [15,31,38]. ADSCs were performance in veterinary stem cell research (such as swine craniomaxillofacial tissue defect, mouse subcutaneous tissue defect, rabbit cartilage tissue engineering and swine full thickness wound defects) for restoring soft tissue defects [39-42].

According to a previous study, dogs are an effective model for studies in tissue engineering and regenerative medicine research because, in addition to presenting structures and functions similar to that of the human, they show similarity in physiological, anatomical and organ characteristics, also in addition to comparable respiratory frequency and social behavior [43,44]. Also, dogs boast a large reserve of adipose tissue, facilitating stem cell isolation from adipose tissue and autologous or allogeneic transplantation, in order to assess the function of these cells in cell therapy. The present study evaluated the efficiency of allogeneic stem cells by injecting different treatment of cADSCs with and without fibrin biomaterial into experimentally created the ear auricle defect in animal models. In the present study, aggrecan and type II collagen gene expression and protein production were shown by Real-time PCR and immunohistochemical examination. The result of real-time PCR and histological analysis showed that cADSCs injected into ear auricle defect by fibrin glue biomaterial had significant potential of regeneration. On the contrary, canine adipose-derived stem cells alone used as control could regenerate significantly smaller cartilage than canine adipose-derived stem cells seeded on fibrin glue scaffold in the this study. In present study, we found that ADSCs-fibrin glue biomaterial injection into dog ear auricle defect after sixty days, can be caused repair on the dog ear auricle defect. The results of our previous study showed that the proliferation and differentiation ADSCs can be increased on fibrin glue biomaterial *in-vitro* and consistent with obtained results in last studies [11]. In addition, many studies were reported the compatibility of fibrin biomaterial in cell therapy and cartilage tissue engineering [10]. Bahrani, et al., illustrated that encapsulation of ADSCs by fibrin biomaterial was capable of healing rabbit ear auricle defects [45]. In the other study, Adipose-derived stem cells were also obtained from inguinal fat pads of rabbits, were differentiated and expanded *in-vitro* and seeded onto 3D biodegradable alginate and silk polymer ear-shaped scaffold [41].

Conclusion

This study demonstrates for the first time that it is possible to engineer an ear cartilage construct that resembles the ear not only in shape, but also in size and flexibility in a “real test” model. For cartilage reconstruction *in-vivo*, canine ADSCs were mixed with fibrin glue biomaterials and were injected into ear auricle defects. After two months, the composites with canine ADSCs generated the native form of cartilaginous tissue as those with ear auricle. Based on these findings, we propose that the canine ADSCs containing tissue progenitor cells are potential candidates for use in cartilage reconstruction and new therapeutic modalities.

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